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The subject of this grant is the development of a novel class of anticancer agents that induces the degradation of specific proteins by causing them to bind in a stable complex with the chaperone molecule Hsp90. This is accomplished by synthesizing hybrid drugs comprised of the Hsp90-binding drug geldanamycin covalently joined to a high affinity ligand for the protein to be degraded. The goal of this grant was to pilot this idea by making geldanamycin-estrogen hybrids to target the estrogen receptor. We have succeeded in synthesizing a family of such hybrids, in determining characteristics of the linker moiety required for activity and in identifying a selective compound with selective cytotoxic activity against ER-containing MCF-7 breast cancer cells.

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**Progress Report**
**Development of Selective Geldanamycin Derivatives**
**PI-Neal Rosen**
**Memorial-Sloan Kettering Cancer Center**

**Grant DAMD17-97-1-7213**

## Introduction

We have shown that ansamycins herbimycin A (HA) and geldanamycin (GM) represent a novel class of drugs that bind to Hsp90 and inhibit protein refolding (1, 2). They bind to a deep, highly conserved pocket in the amino-terminal portion of Hsp90 (2) (Figure 1). The endogenous ligand that binds to this pocket is unknown: the pocket is homologous to the helicase ATP-bind site and binds ADP and ATP with low affinity (3, 4). The result of ansamycin binding is the selective degradation of a small subset of cellular proteins including transmembrane tyrosine kinases, Raf, and steroid receptors (5-8) (Figure 2). Degradation is ubiquitin- and proteasome dependent (5). The protein targets are generally signaling proteins that require Hsp90. The most sensitive targets we have identified are the HER-family of tyrosine kinases (7, 9) (Figure 2).

The issues we are currently addressing include definition of the cellular effects of the drug and determination of whether regulation of Hsp90 function plays a role in physiologic regulation of signal transduction and whether ansamycins can be exploited clinically.

This grant deals with the latter issue.

## Results

We have considered whether ansamycins could be used as anticancer drugs. They are potent inhibitors of cancer cell growth, but their mechanism of action suggests that they would be quite toxic. In limited studies in tissue culture, tumor cells are more sensitive than untransformed cells. HA has been used effectively in several animal leukemia models (10, 11). We have conceived of three possibilities for their use in the clinic.

**1. Treatment of tumors that depend on a target that is very sensitive to GM.** These would include tumors in which the HER2 gene is amplified and those containing HER2/HER3,4 heterodimers. These might include advanced, hormone-independent prostate cancers that require HER2 or mutated, activated androgen receptor. Each of these proteins is a sensitive target of GM action and prostate cancer cell lines expressing these proteins are inhibited and killed by nanomolar concentrations of this drug. An NCI-sponsored phase I trial of 17-allyaminoGM in which we plan to participate will begin this winter.

**2. Use in combination with radiation or cytotoxic chemotherapy.** Inhibition of protein refolding by these drugs suggests that they could be used as radiation of chemotherapy sensitizers. Animal and cell culture models are being used to test this possibility.

The third possibility is the subject of this grant:

3. In collaboration with Dr. Samuel Danishefsky we have proposed that **derivatives of ansamycins that induce the selective degradation of specific proteins can be synthesized**. These molecules would consist of ligands that bind with high affinity to targeted proteins linked covalently to an ansamycin. The hybrid drug would induce formation of a stable complex comprised of Hsp90, drug and target protein. This complex would be inactive enzymatically (in the case of steroid receptors) and a target for degradation in the proteasome.

There are two levels to this strategy. First, proteins that are sensitive to the parent compound can be targeted. The goal is to create an agent that has selective potency against that protein compared to other targets of GM. Such an agent would presumably be less toxic than GM. Thus far, we have synthesized and characterized three drugs with relative selectivity targeted to HER-kinases, estrogen receptor and androgen receptor, respectively (See below.) Second, proteins that are insensitive to the parent compound can be targeted. The hope is that any heterodimer of Hsp90 and a particular protein will be inactivated enzymatically and, perhaps, subject to degradation. We are in the process of synthesizing GM-hybrids targeted to PI3 kinase and to various immunophilins. These syntheses are in progress; lead compounds have not been identified.

Another strategy is based on the fact that transmembrane tyrosine kinases heterodimerize when activated. We synthesized GM dimers joined at the 17-position by linkers of various lengths. The ideas is that some selectivity might be achieved as a function of type and length of linker. This strategy has proven to be successful (See below.)

**Technical Objectives 1 and 2** in the grant are the synthesis and biochemical testing of hybrid drugs targeted to important proteins in breast cancer. We have managed to synthesize three families of molecules; estradiol-linked geldanamycins, testosterone-linked geldanamycins, and geldanamycin dimers attached with a series of different length linkers. We have determined some parameters of linker length and composition that correlate with potency and selectivity. We have isolated geldanamycin-estradiol hybrids with specificity for estrogen receptor and HER2 and geldanamycin dimers selective for HER kinases. The testosterone-geldanamycin hybrids we have synthesized have only weak activity in inducing degradation of any target, but they are quite potent and selective inhibitors of the growth of tumors containing wild type or mutant androgen receptor. We have therefore speculated that this drug induces the formation of a stable Hsp90-androgen receptor complex that is not degraded but can no longer be activated. This assertion is being tested.

We have also now synthesized geldanamycin derivatives targeted to PI3 kinase in an attempt to generalize the concept to target proteins that are insensitive to the parent compound. Biochemical testing of these compounds is just beginning.

**Technical Objective 3.** Assessment of the effects of the hybrid drugs on breast cancer cells. Our current best compounds are:

1) a GM dimer that potently inhibits the growth of HER-kinase containing breast cancer cells;

2) a GM-estradiol hybrid that is 10-15-fold less active than GM in MCF-7 cells, but that shows selectivity towards degradation of the ER; and
3) a GM-testosterone hybrid that is a potent inhibitor of prostate cancer cells with wild type or mutated androgen receptor ($IC_{50}$ ~ 10-20 nM) and much less active against cell lines without receptor ($IC_{50}$ greater than 500 nM).

**Properties of specific GM-derivatives**

**A. Estrogen-geldanamycin hybrids.** Several E2-GM dimers have been synthesized. Activity is highest with unsaturated linkers 4-6 carbons long (Table I) (12). One of these has been selected as a lead compound (II-211, Figure 3). It is 10-20 fold less potent than geldanamycin against estrogen receptor, but 200-1000 fold less potent against androgen receptor, IGF-I kinase and Raf (Figures 4 and 5). It still induces HER2 kinase degradation with the same potency as displayed against estrogen receptor (12). Thus, this represents a partially selective compound. It may be useful for the treatment of breast cancer, as both HER2 and estrogen receptor are key targets in this disease (13-16). Preliminary testing of the drug shows that, although it is less active than GM, it is cytotoxic to several breast cancer cells that contain estrogen receptor (Figure 6).

**B. GM-dimers.** A class of GM-dimers joined at the 17-carbon by linkers varying in length from three to twelve carbons were synthesized (Figure 7). The ability of these dimers to induce the degradation of a collection of GM-target proteins was evaluated. The dimers were less potent than GM or 17-allylamino-GM and potency declined with increasing linker length (Table II). The 4-carbon linked dimer (4C-GM, II7 or IIn1) was selective for HER-family tyrosine kinases. It was much less active against Raf and steroid receptors and inactive against the IGF-I receptor (Table II and Figure 8). Selectivity was not secondary to the isolation of a generally weak agent, which retained activity only against the most sensitive target(s). Selectivity was maintained when the drug was given frequently and at high concentration (Figure 9) (7). Furthermore, selectivity required intact dimer. Selectivity was lost when both or either of the ansa rings was opened (II-13F1 and II-13F2, Table II).

Identification of a HER kinase selective drug was of great interest, given the proven role of these enzymes in multiple tumors and the known sensitivity of breast cancers with HER2 gene amplification to an anti-HER2 antibody (13-16). Accordingly, we have begun to the effects of 4C-GM on cancer cells. The drug is a potent inhibitor of the growth of breast cancer cell lines with HER2 kinase (7) (Figure 10). As opposed to GM, 4C-GM causes a much more specific G1 block in Rb-positive cells (Figure 11). Growth inhibition is followed by either cell death and/or differentiation (Figure 12). Growth inhibition is accompanied by loss in HER-kinase expression but not in loss of the expression of other GM targets (Figure 8). Taken together, the data suggests that this drug is more selective than GM, potently inhibits tumor cell growth, and is likely to have fewer side effects.

The properties of this drug and the importance of its target have led us to identify 4C-GM as a lead compound and to put high priority in its development. Current goals include studies on the biochemical mechanism of action, testing of the drug against breast cancers in HER2 transgenic mice and the synthesis and testing of other GM-dimers with different linkers.

**C. Testosterone-GM hybrids.** Similar testosterone-GM hybrids were synthesized (Figures 13 and 14) (8). These were much less potent than either GM or the estrogen-GM in inducing protein degradation (Figure 15). However, one testosterone-GM hybrid (GMT1) was a potent and selective inhibitor of prostate cancer cell growth (8). It inhibits the proliferation of tumor cells with wild type or mutated androgen receptor at doses much (30-100-fold) less than those required to inhibit cells without the receptor (Figures 16 and 17, Table III). Furthermore, inhibition occurs in the absence of degradation of families of protein targets (Figure 15). These data suggest that this drug will be a potent inhibitor of advanced prostate cancer with much less toxicity than the parent compound. We do not yet understand the mechanism of this effect, but we speculate that this GM-hybrid prevents the dissociation of androgen receptor from Hsp90 and thus suppresses the activity of the androgen receptor.

We regard this compound as a very exciting discovery that may be useful in the treatment of prostatic cancer. We now plan to synthesize a family of related derivatives, investigate the mechanism of selective growth inhibition and assess these compounds in animal models of advanced prostate cancer.

**Other work**

One might expect that a drug that regulates the activity of a chaperone with housekeeping function would have global effects on protein expression and cause non-specific cellular toxicity. This is not the case. Addition of GM or HA to cells results in degradation of a small, selected subset of proteins (5). The general profile of cellular protein expression is unaffected. Nuclear steroid receptors and members of the human epidermal growth factor receptor (HER)-family tyrosine kinases are the most sensitive targets we and others have observed (5, 7, 17-19) (Figure 2). Some of the other targets for GM include other receptor tyrosine kinases (5), Src family members (20); Raf kinase (21, 22) and mutant p53 (23-26). Degradation is ubiquitin- and proteasome dependent (5). The protein targets are generally signaling proteins that require Hsp90. The most sensitive targets we have identified are the HER-family of tyrosine kinases (7, 9) (Figure 2). The results suggest that Hsp90 may play some specific role in stabilizing certain proteins involved in transducing the mitogenic signal or in regulating their function.

This view was reinforced when the effects of ansamycins on cells were studied. Addition of these drugs to tumor cells leads to growth arrest and subsequent apoptosis and or differentiation. The induction of profound differentiation by HA in several systems again suggested that the drug was regulating specific pathways. Analysis of the mechanism of growth arrest confirmed this supposition. Ansamycins caused most cancer cells to arrest in the G1 phase of the cell cycle (27-29). We demosntrated that the G1 block is Rb-dependent. Rb-negative cells traverse G1 normally in the presence of ansamycins. Instead, they arrest in early anaphase (28). Transfection of RB in Rb-negatives reverses this phenotype. Transfection of the papilloma virus E7 gene into Rb-positive cells were arrested in anaphase and not G1. This means that the effects on G1 progression of ansamycin targets, which include multiple steroid and transmembrane tyrosine kinase growth factor receptors as well as Raf, are confined to the D-cyclin-cdk-Rb axis. Second, these results suggest that Rb plays a role in the regulation of mitotic progression or of a mitotic checkpoint. The possibility that the M-block is due to a target that is degraded slowly,

so it is not observed in cells that can block in G1 was ruled out. When Rb positive cells in S phase were treated with ansamycins, they arrested in the next G1; Rb negative cells treated similarly blocked earlier, in the next anaphase (28).

Since our results indicated that the reduction in D-cyclin expression induced by ansamycins was an important determinant of G1 block, we explored its mechanism (27). We found that ansamycins decreased and serum and growth factors increased the synthesis of D-cyclins via a PI3kinase- and Akt- dependent pathway (27). Overexpression of myristylated, active Akt kinase prevents the effect of ansamycins on D-cyclins (27). Thus, growth factors stimulate D-cyclin expression by enhancing translation of the mRNA via a pathway mediated by PI3-kinase and Akt and ansamycins antagonize this pathway. The mechanism of antagonism has not been rigorously proven but likely involves destruction of upstream receptor tyrosine kinases. We have also shown that ansamycins decrease the levels of expression of Akt kinase (27). Whether this is a primary effect of the drug on Akt or whether inhibition of the pathway leads to a decline in Akt protein is under investigation.

## Plans for this year

- We will continue our original plan. New hybrid molecules with different linkers will be synthesized to attempt to improve potency and selectivity of estrogen receptor, HER kinase and testosterone receptor activity.
- The first generation of compounds targeted to PI3-kinase will be evaluated.
- The mechanism of biochemical and cellular specificity will be addressed.

In other work, not funded by this grant, clinical testing of the parent compound, geldanamycin, will begin. It turns out that geldanamycin is quite toxic to animals. This extreme toxicity is mediated by Hsp90 independent mechanisms, The derivative 17-allylaminogeldanamycin does not share this toxicity but binds to Hsp90 and affects tumor cells in a fashion similar to GM. In collaboration with E. Sausville and S. Arbuck at the NCI, we will test this agent clinically in the spring of 1999.

## Conclusions

We have successfully synthesized a family of geldanamycin-estradiol hybrid drugs attached by linkers with different properties. We have isolated partially selective drugs that degrade estrogen receptor and HER2 but are much less active against other geldanamycin targets including IGF-I receptor, Raf, and androgen receptor. We regard this as proof of principle of original concept: that geldanamycin derivatives could be synthesized with different specificity than that of the parent molecule. Furthermore, we predict that the molecule we have synthesized will be less toxic than the parent and a potentially good drug for breast cancers expressing estrogen receptor, with or without HER2. We have now synthesized enough drug to test in animals. These studies are beginning in several weeks.

We have extended this work to synthesize an analogous testosterone-geldanamycin hybrid. This drug potently inhibits androgen receptor containing prostate cancer cell lines selectively; it is much less active (100-fold) in tumor cell lines that don't contain the receptor.

In other studies, not part of this grant, we have characterized some of the mechanisms whereby geldanamycin kills tumor cells. We plan, in collaboration with the NCI, to begin a phase I study of 17-allyaminogeldanamycin this spring. This will be the first clinical study of this class of drugs.

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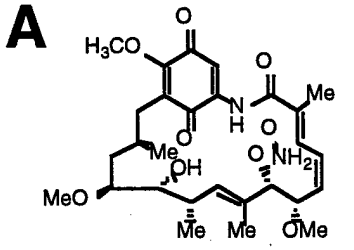
# Appendix

## Figures

**Figure 1. A) Structure of geldanamycin. B) Hsp90 GM-binding domain pocket.**




Geldanamycin (GM)

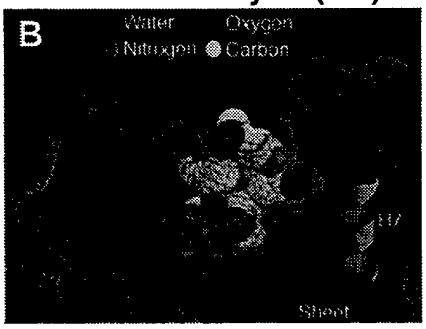



**Figure 2. Changes in protein expression induced by GM.** Most cellular proteins are not affected, heat shock proteins are upregulated. A specific set of proteins are decreased in response to GM. Decrease in steady-state levels in most cases, is mediated by enhanced degradation.

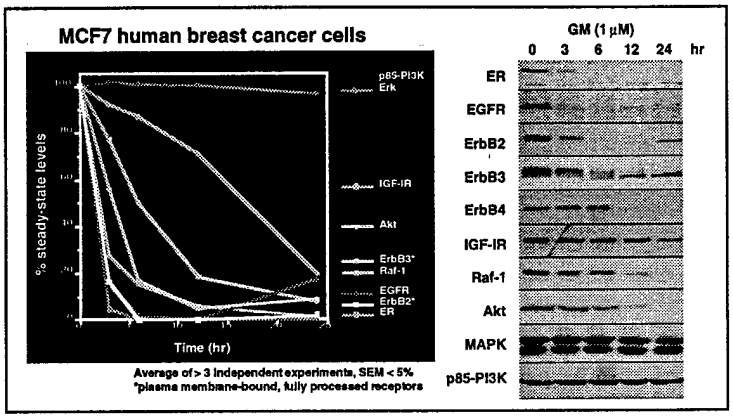



Table I: Effects of GM-Estradiol hybrid molecules on the steady-state levels of HER2, ER and Raf-1 in MCF7 cells

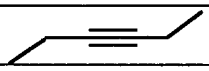
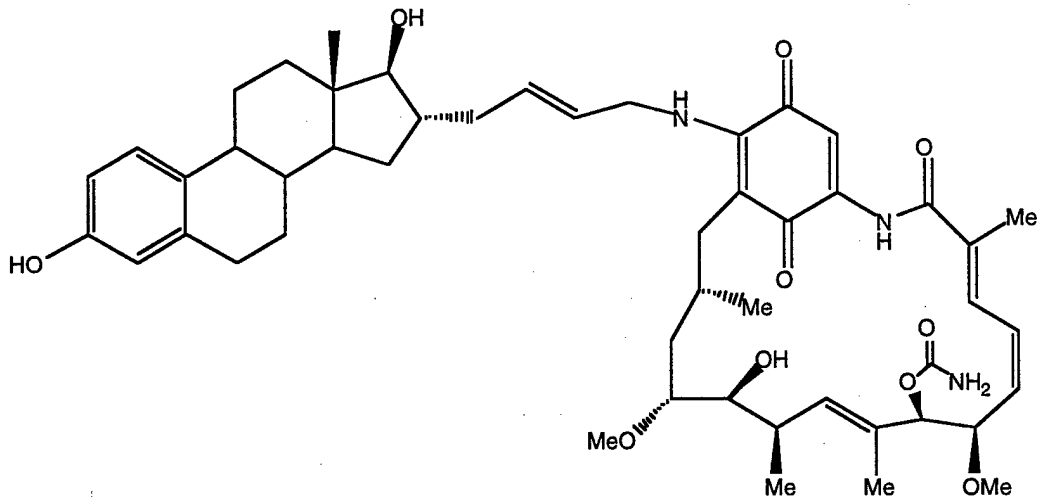
| Compound | LINKER | ErbB2 degradation $IC_{50}$ (nM) | ER degradation $IC_{50}$ (nM) | Raf-1 degradation $IC_{50}$ (nM) |
|---|---|---|---|---|
| GM | – | 45 | 60 | 200 |
| GM-E2 A<br>II-211 |  | 100 | 80 | 1,500 |
| GM-E2 B |  | 100 | 220 | 1,500 |
| GM-E2 C |  | >2,000 | >2,000 | >2,000 |
| IGM-E2 B |  | >2,000 | >2,000 | >2,000 |

**Figure 3. Chemical structure of geldanamycin-estradiol hybrid II-211**




**II-211**

**Figure 4. The GM-E2 hybrid compound II-211 induces ER and Neu degradation, but has no effect on other GM targets, such as Raf-1 and the IGF-I receptor.** MCF7 cells were treated with 1 uM drug for increasing time periods and proteins were analyzed by immunoblotting with specific antibodies. Expectedly, estrogen induced the downregulation of its own receptor, but had no effect on the steady-state levels of other proteins.

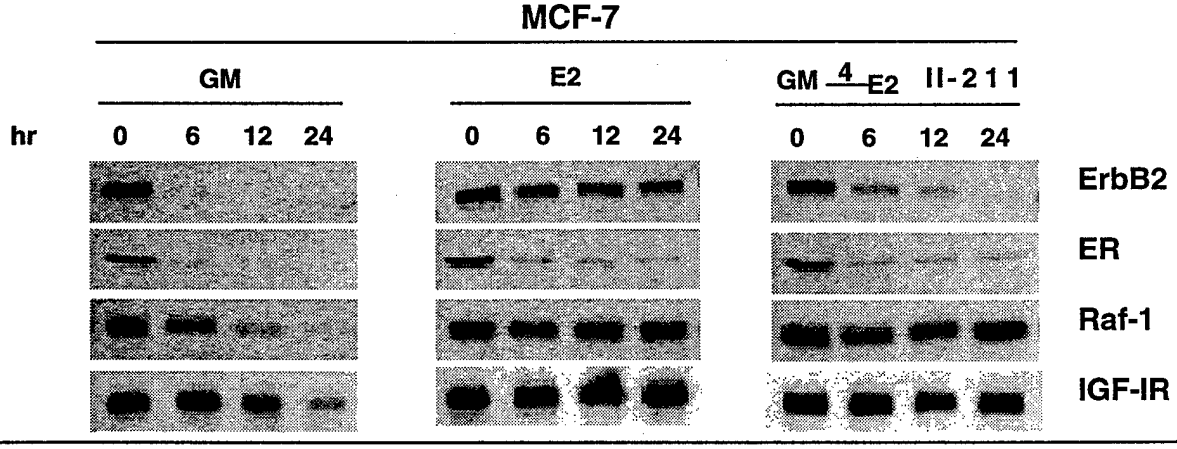


**Figure 5. The GM-E2 hybrid compound II-211 has no effect on the levels of androgen receptor, while still induces the degradation of ErbB2.** LNCaP human prostate cancer cells were treated with 1 uM drug for increasing time periods and proteins were analyzed by immunoblotting with specific antibodies. Neither dehydrotestosterone (DHT), nor estrogen (E2) had any effect on the levels of the androgen receptor or of any other protein.

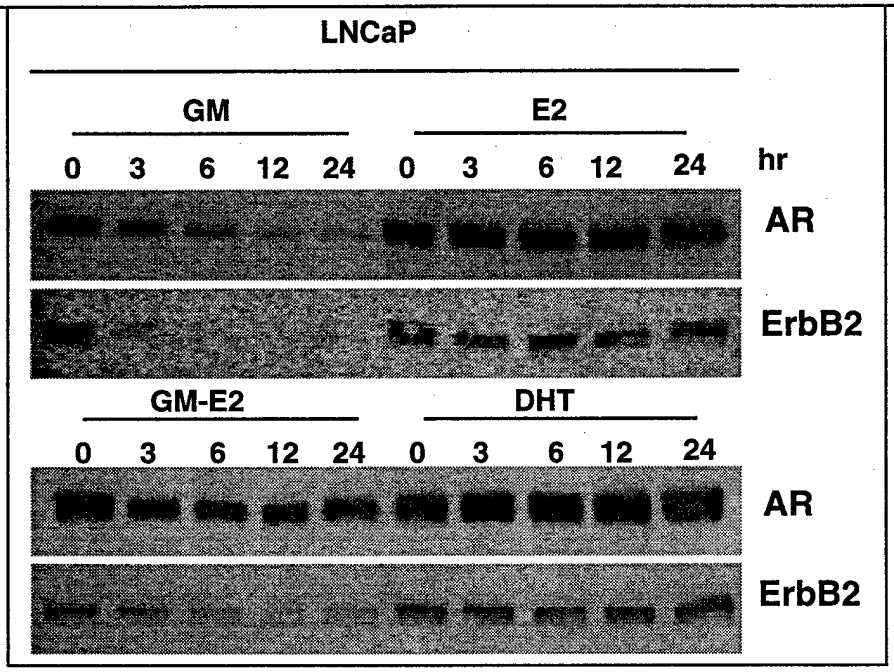
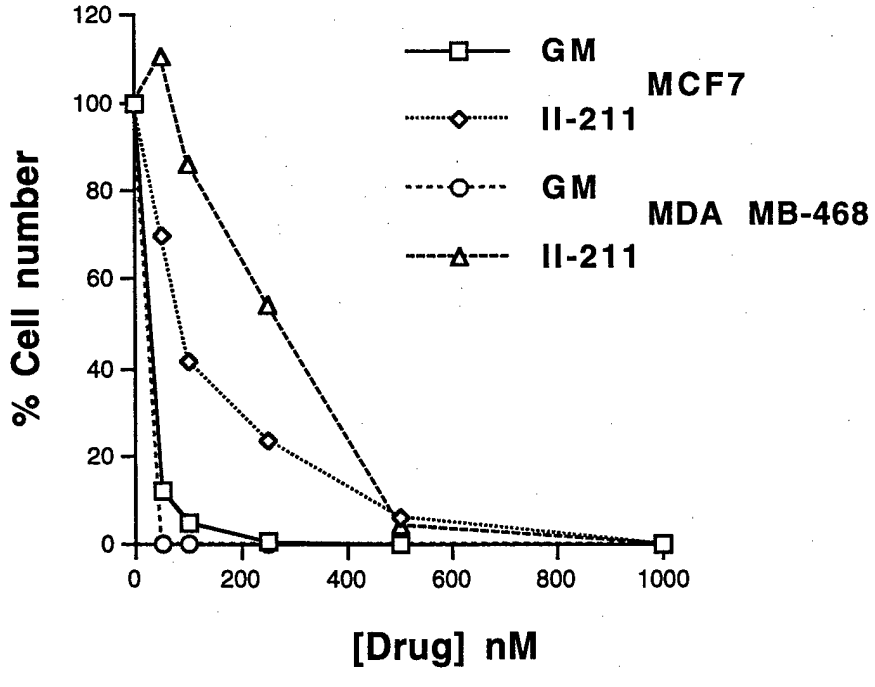
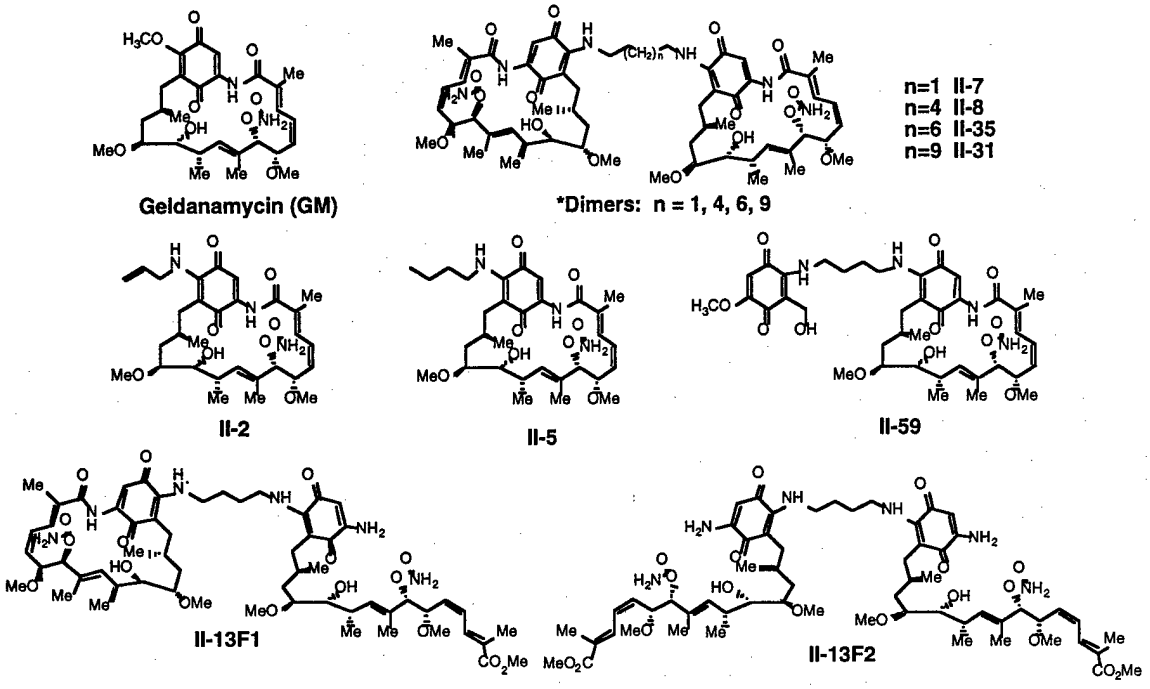



**Figure 6. The GM-E2 hybrid compound II-211 inhibits the growth of human breast cancer cells.** MCF7 and MDA MB-468 human breast cancer cells were treated with increasing drug concentrations. Cell number was determined after 5 days.



## Figure 7. Chemical structure of geldanamycin dimers




## Table II: Effects of GM analogs on cell growth and protein levels in MCF7 human breast cancer cells

| Compound | ErbB2 degradation $IC_{50}$ (nM) | Raf-1 degradation $IC_{50}$ (nM) | Growth inhibition $IC_{50}$ (nM) |
|---|---|---|---|
| GM | 45 | 200 | 25 |
| II-7/ IIn1 4C-dimer | 60 | 3,200 | 100 |
| II-13F1 one ring open | 500 | 3,500 | 650 |
| II-13F2 two rings open | >10,000 | >10,000 | >10,000 |
| II-8 | 70 | 500 | 600 |
| II-35 | 500 | 3,800 | 700 |
| II-31 | 700 | 3,500 | 700 |
| II-5 | 80 | 350 | 600 |
| II-59 | 55 | 350 | 350 |
| II-2 | 50 | 250 | 80 |

**Figure 8. Effect of GM and GM-4C (II7, IIn1) dimer on the steady-state of MCF7 cellular proteins.** MCF7 cells were treated for increasing time periods with 1 uM drug. Cells were harvested and SDS-protein extracts were analyzed by SDS-PAGE. The steady-state levels of several GM-targets were analyzed by immunoblotting with specific antibodies.

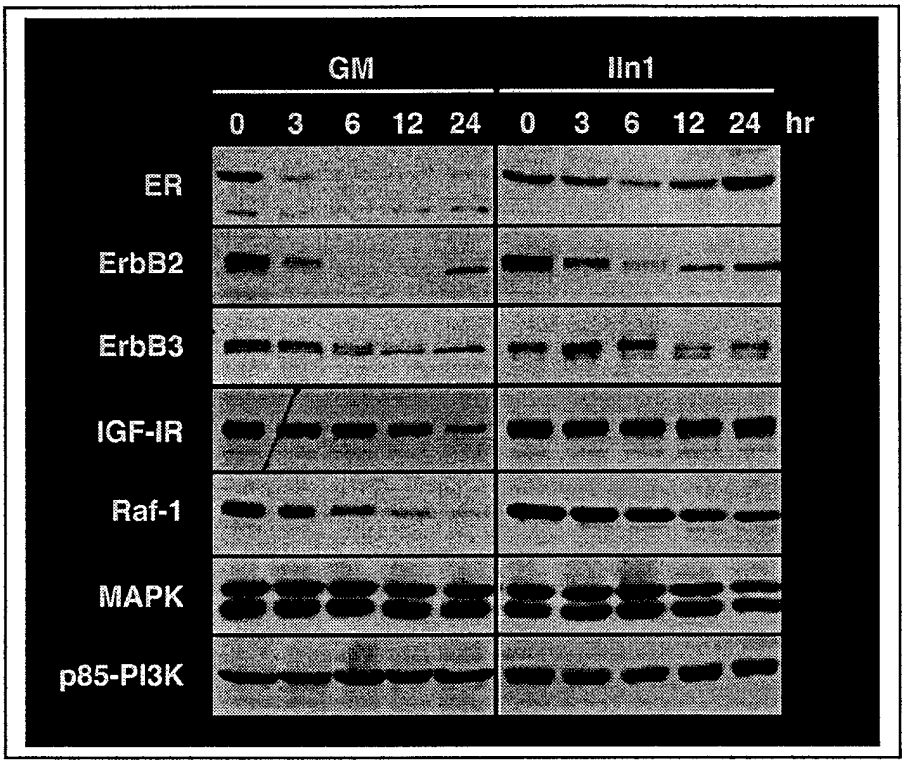



**Figure 9. Selective effect of GM-4C (II7, IIn1) dimer is unrelated to changes in its intracellular uptake or half life.** MCF7 cells were treated with GM or dimer for a total of 12 hr. Cultures received drug either once, twice or every 3 hr until completion of the 12 hr period. Cells were harvested and Raf and Neu levels were analyzed by immunoblotting,

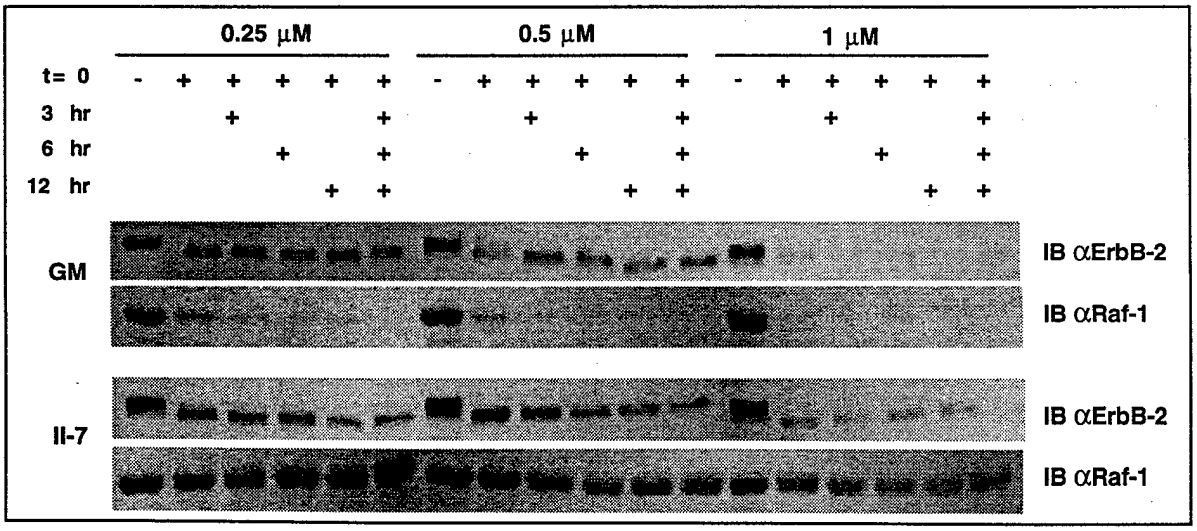
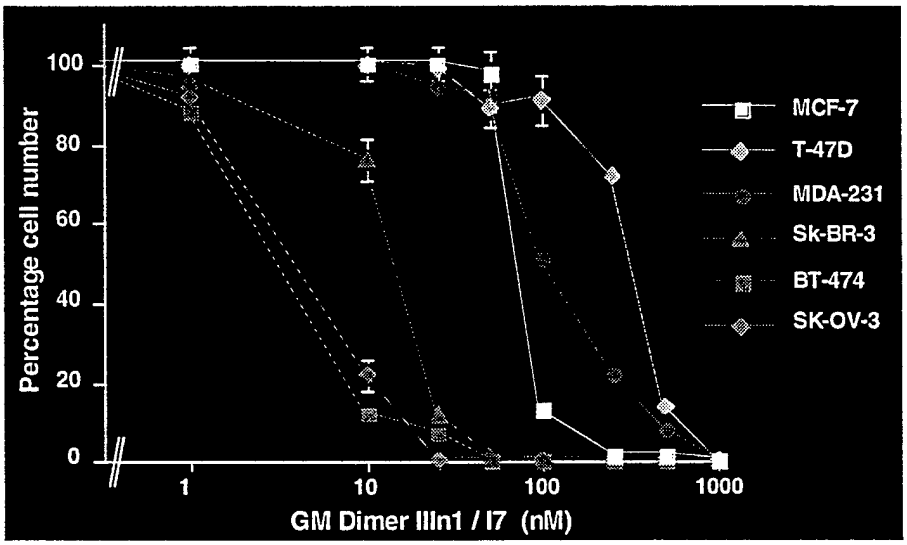


**Figure 10. 4C-GM dimers are most effective against HER2 overexpressors.** A variety of breast cancer cell lines expressing different levels of HER2 were tested for sensitivity to the 4C-GM dimer. Cells were grown in the presence of drug or DMSO for 5 days, when cell number was determined. Inhibitory concentrations that induce over 95% cell growth inhibition compared to DMSO controls (IC95) were calculated and are shown below. Data on HER2 expression was obtained from the literature and/or determined in our laboratory.




| Cell line | BT-474 | SKBr3 | SKOv3 | MCF7 | MDA-231 | T47D |
|---|---|---|---|---|---|---|
| HER2 gene copy number | 52 ± 11 | 31 ± 9 | | 2.2 ± 0.5 | | |
| HER2 protein expression | +++ | +++ | +++ | + | + | + |
| GM dimer Conc. (nM) for <95% inhibition | 10-25 | 10-25 | 10-25 | 100-250 | 500 | >500 |

**Figure 11. 4C-GM dimers induce G1 arrest.** FACS analysis of MCF7, SKBr3, BT474 and MDA 231 breast cancer cells treated with increasing GM dimer concentrations.

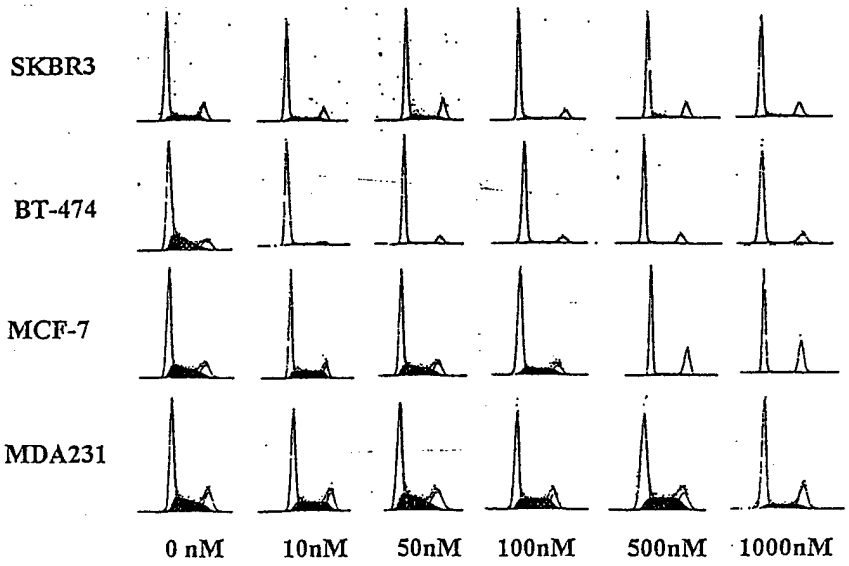


**Figure 12. Breast differentiation induced by GM-4C (II7, IIn1) dimer.** MCF7 cells were treated with drug or DMSO for 0-96 hr. **A)** Oil-Red-O stain for fat globules after 96 hr treatment (40X). **B)** Production and secretion of fat milk globulin (Human HMFG).

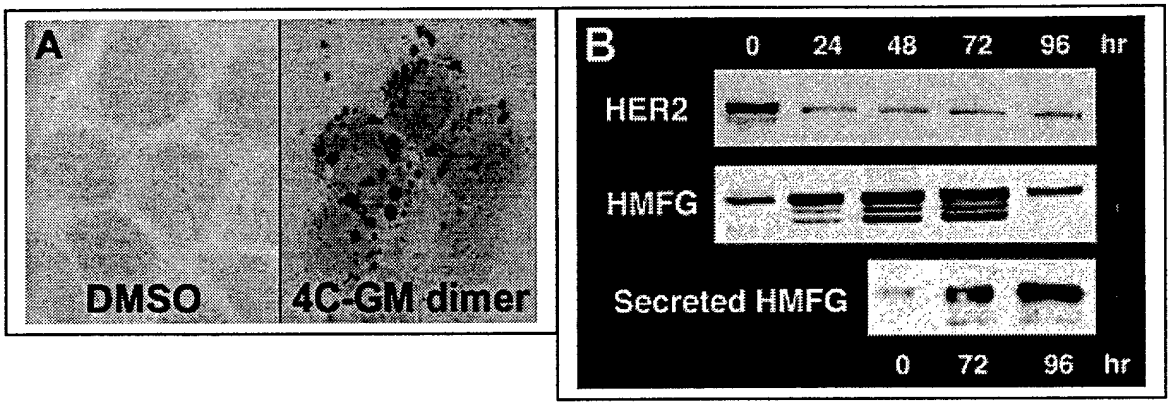



**Figure 13. Structures of GM-dehydrotestosterone hybrids.**

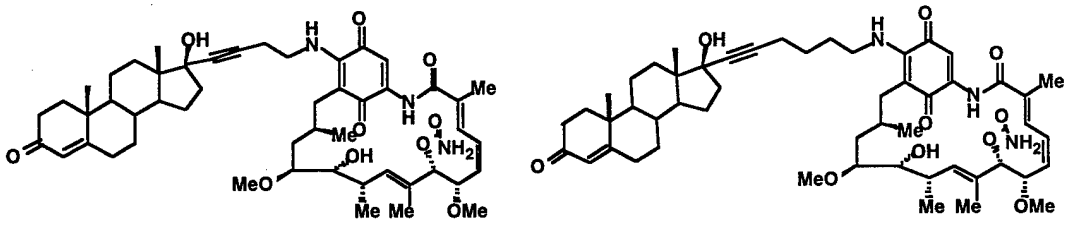



**Figure 14. Synthesis of GM-dehydrotestosterone hybrid II-298.**

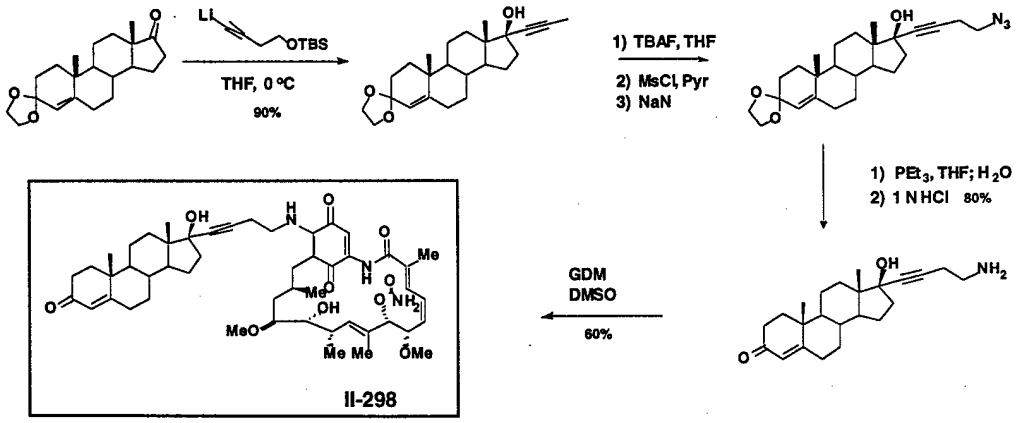


**Figure 15. Effect of GM and GM-testosterone hybrids on the steady-state levels of cellular proteins on LNCaP human prostate cancer cells.** A) LNCaP cells were grown in media supplemented with 1 uM GM, GM-DHT hybrids or dehydrotestosterone (DHT) for 0 to 48 hr. Cells were harvested and proteins were analyzed by immunoblotting using specific antibodies for androgen receptor, HER2/Neu or Raf-1. B) LNCaP cells were treated for 48 hr with various concentrations of GM, GM-DHT hybrids or DHT. Protein levels were analyzed as described for A).

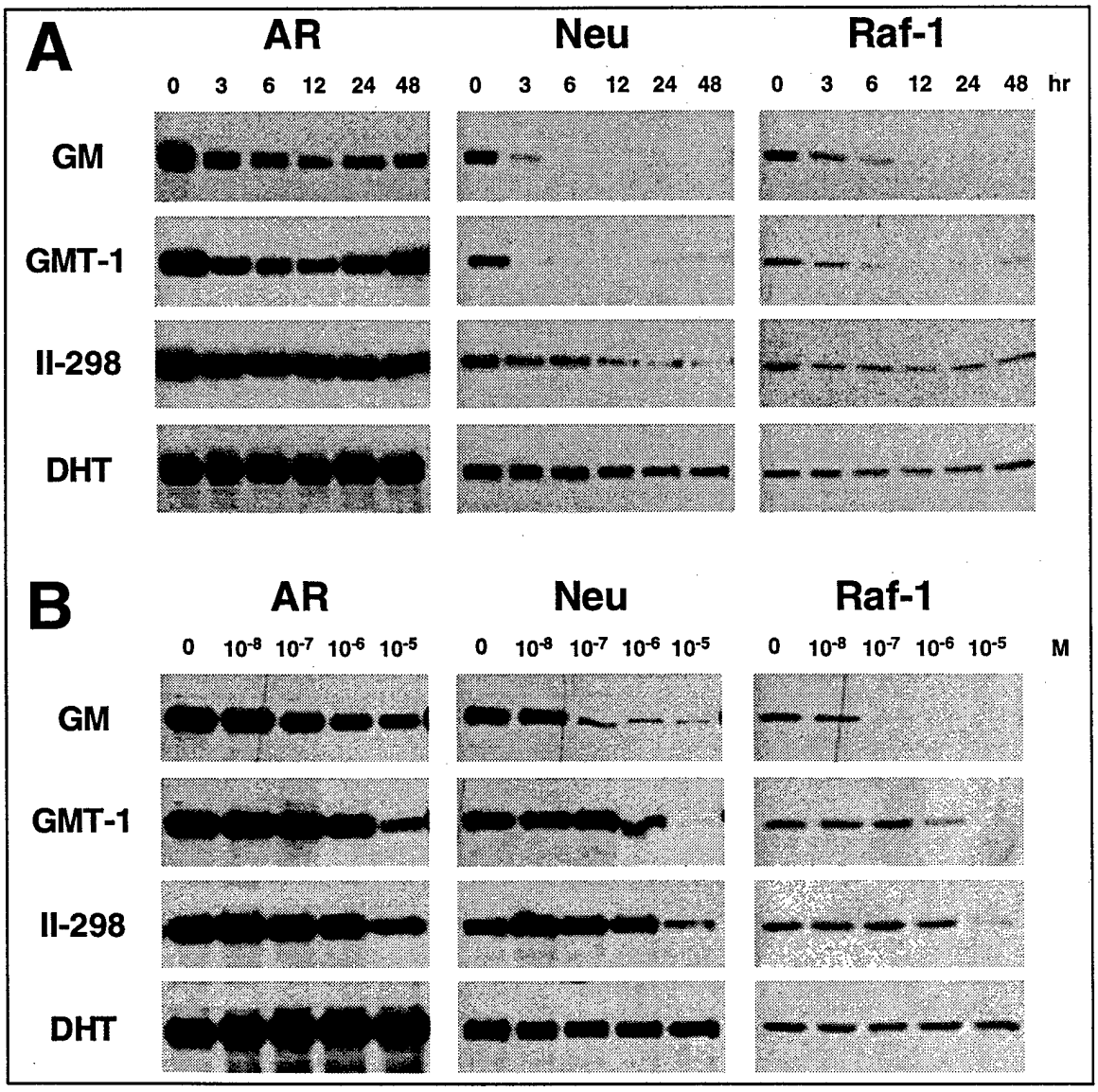


**Figure 16. The GM-testosterone hybrid GMT1 is selectively cytotoxic against AR-positive cells.** IC50 values for GM and GMT1 were determined for AR-positive (LNCaP) and negative cells (MCF7) cells.

| GM | $IC_{50}$ (nM) | GMT1 | $IC_{50}$ (nM) |
|---|---|---|---|
| LNCaP | 40 | LNCaP | 75 |
| MCF7 | 30 | MCF7 | 500 |

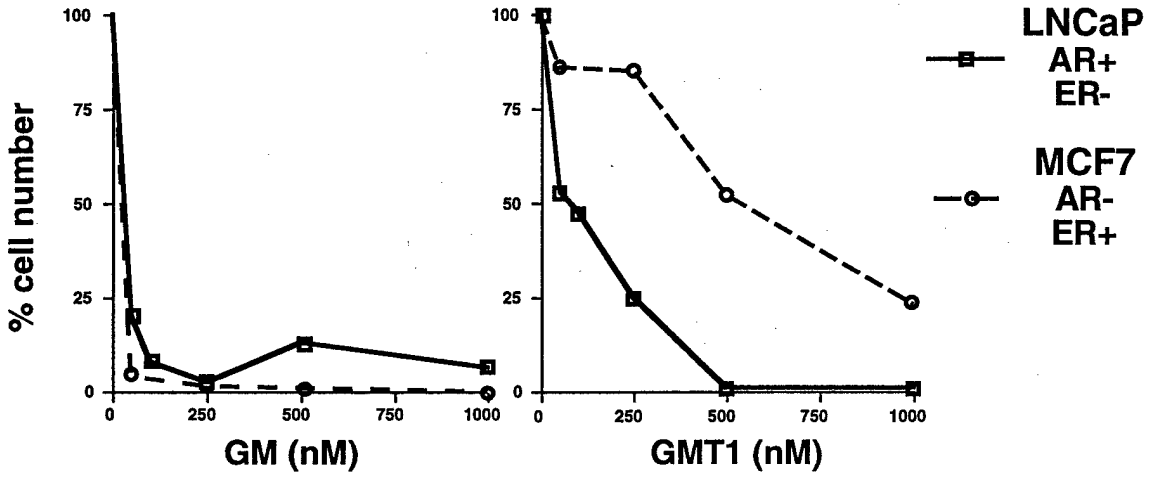



**Figure 17. Growth curves for GM and the GM-testosterone hybrid GMT1 for AR-positive LNCaP cells and for AR-negative MCF7 cells.**

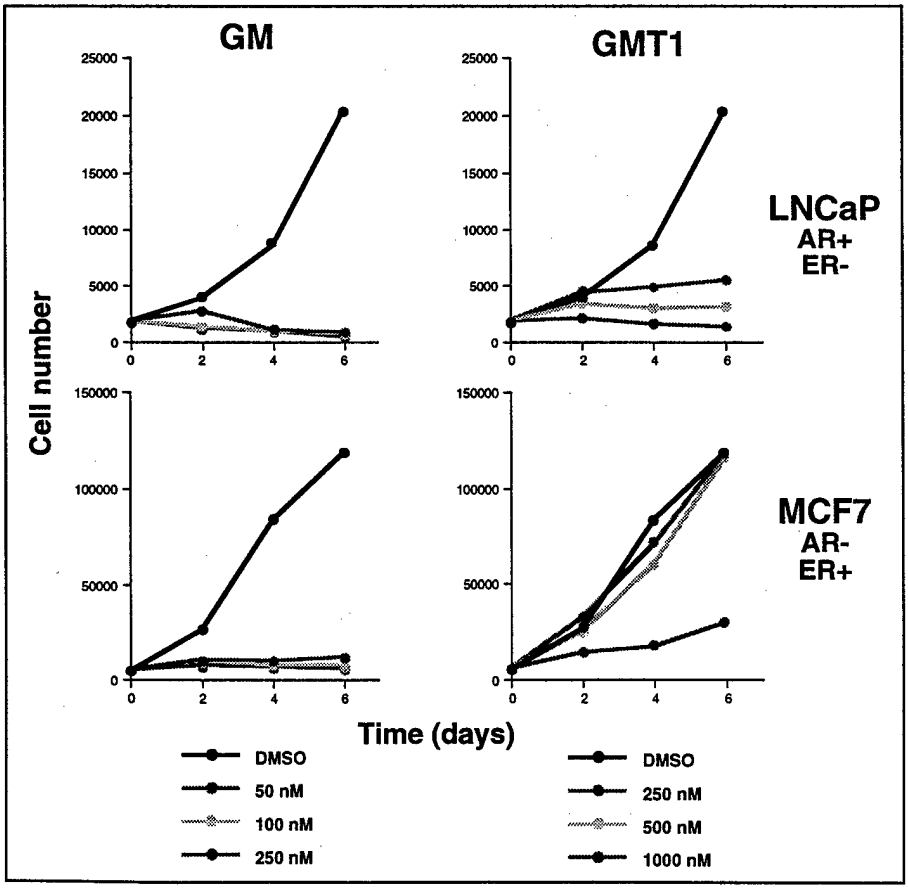


**Table III - Increased cytotoxicity of the DHT-GM hybrid molecule GMT1 towards androgen receptor positive cells.**

| Cell line | Origin | Steroid receptor | GM | GMT1 (GM-DHT) |
|---|---|---|---|---|
| LNCaP | Prostate Ca. | AR + | 40 | 75 |
| LAPC4 | Prostate Ca. | AR + | 18 | 30 |
| PC3 | Prostate Ca. | AR - | 20 | 270 |
| DU145 | Prostate Ca. | AR - | 30 | 160 |
| MCF7 | Breast Ca. | ER + | 30 | >500 |
| MDA MB-468 | Breast Ca. | ER - | 20 | 190 |
| Colo 205 | Colon Ca. | None | 20 | 170 |